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# *Pseudomonas syringae* pv. *actinidiae* strains isolated from past and current epidemics to *Actinidia* spp. reveal a diverse population structure of the pathogen

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**Abstract** A total of 40 *Pseudomonas syringae* pv. *actinidiae* (Psa) strains isolated from past and current epidemics of kiwifruit bacterial canker worldwide were compared using repetitive-sequence PCR (rep-PCR) fingerprinting with BOX, ERIC and REP primer sets. The strains were also assessed for the presence of 23 type III secretion system effector genes, tolerance to sodium arsenite, the presence of coronatine and phaseolotoxin and for growth trends in *Actinidia deliciosa* leaves. Rep-PCR revealed the occurrence of 11 different Psa lineages among the strains and indicated a relevant genetic variability within the strains isolated in Japan during 1984–2011, whereas all strains obtained from the current pandemic showed the same banding pattern. All lineages showed the same growth trend when inoculated into *A. deliciosa* leaves. The difference between Psa strains from past and current epidemics was confirmed by the detection of different repertoires of type III effector protein genes. Not all Psa strains isolated in Japan during past epidemics of kiwifruit bacterial canker amplify genes of the *argK-tox* cluster of phaseolotoxin, suggesting their absence or gene mutation. The results of an arsenic tolerance assay indicated that almost all strains isolated in Italy during the current epidemic of kiwifruit bacterial canker were relatively tolerant to 0.15–0.35 mM sodium arsenite, whereas those isolated in Chile and New Zealand were very sensitive. Remarkably, the two strains from China were tolerant or very sensitive. Collectively, these data indicate a composite population structure of this pathogen, which was able to diversify in Japan during 27 years of recurrent infections to *A. deliciosa*. The current naming of Psa populations based on their numbering and presence/absence of phytotoxins should be reconsidered.

**Keywords** Kiwifruit bacterial canker · Repetitive-sequence PCR · Type III secretion system effectors · Pandemic · Phaseolotoxin

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## Introduction

The determination of a pathogen population structure is a crucial step for predicting the genetic diversity of the pathogen, the origin of an epidemic, the spread of clonal lineages, the emergence or re-emergence of new populations, the impact and effectiveness of control measures (Hallin et al. 2012) and the suitability of current phytosanitary pathogen detection protocols (Ferrante and Scortichini 2015). The relationship between pathogenic bacteria and the host plant(s) involves adaptation to the particular growth environment, the commercial

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trade routes of individual plant parts (i.e., seeds, bulbs, or scions), and the subsequent material uses, which leads to intricate and multifaceted opportunities for global spreading and diversification; thus, commercial agricultural plant-pathogen phylogenetic analysis offers a prolific model for assessing the structure of evolutionary time and space in host-pathogen relations (Scortichini 2005).

Analyses of *Pseudomonas syringae* pathovars (pvs) have successfully provided information on population structure and uncovered relevant genetic diversity. In fact, *P. s.* pvs *lachrymans*, *phaseolicola*, *pisi* and *syringae* strains have been assessed using various molecular fingerprint-based (i.e., repetitive-sequence PCR, PFGE, AFLP, RFLP, and IS) or sequence-based (i.e., MLST and ITS) techniques. These analyses have revealed different clustering patterns (Scortichini et al. 2003; Oguiza et al. 2004; Cirvilleri et al. 2007; Olczak-Woltman et al. 2007; Kaluzna et al. 2010; San Josè et al. 2010; Martin-Sanz et al. 2012; Gutiérrez-Barranquero et al. 2013; Slomnicka et al. 2015). Sometimes, the clustering patterns were clearly related to a different virulence factor toward the host plant (Cirvilleri et al. 2007; Slomnicka et al. 2015) or the presence of a phytotoxin (Gutiérrez-Barranquero et al. 2013).

*P. s.* pv. *actinidiae* (Psa) is the widespread pathogenic agent of bacterial canker in the green-fleshed kiwifruit (*Actinidia deliciosa*) and the yellow-fleshed kiwifruit (*A. chinensis*) that is a currently causing severe economic losses in these worldwide cultivated crops (Scortichini et al. 2012). The Psa genetic structure has been investigated, and a number of populations have been recognized. The population Psa 1 includes the pathovar type-strain NCPPB 3739$^{T}$=KW 11, which was isolated from *A. deliciosa* in Japan in 1984, (Takikawa et al. 1989) and two strains isolated in central Italy in 1992 from the same host plant (Scortichini 1994). Psa 1 strains are characterized by the presence of the phytotoxin phaseolotoxin (Sawada et al. 2002; Ferrante and Scortichini 2010). Psa 2 includes strains isolated solely in South Korea during the 1990s and is characterized by the presence of the phytotoxin coronatine (Han et al. 2003). Psa 3 is the population responsible for the current pandemic in all major areas of kiwifruit production in the world (i.e., China, Europe, New Zealand, Chile, and Japan) (Shinozaki et al. 2014). Psa 3 lacks the genes involved in phaseolotoxin and coronatine production (Marcelletti et al. 2011). In addition, a new lineage has recently been described in Japan that is different from Psa, namely, Psa 5 that causes damage to *A. chinensis* and lacks genes involved in production of coronatine and phaseolotoxin (Sawada et al. 2014). Another similar but genetically different population, which was previously claimed to belong to the Psa 4 or Psa-less virulent lineage (Chapman et al. 2012; Vanneste et al. 2013), has been shown to not belong to pv. *actinidiae* (Ferrante and Scortichini 2015). This lineage was proposed to represent a new *P. syringae* pathovar, namely, pv. *actinidifoliorum* (Cunty et al. 2014). Recently, it has been shown that *P. syringae* pvs *actinidiae* and *actinidifoliorum* are members of *P. avellanae* genomospecies (Scortichini et al. 2013; Marcelletti and Scortichini 2014).

The aforementioned studies have mainly compared strains of the current pandemic (i.e., 2008 onward), which show a very low genetic diversity (Marcelletti and Scortichini 2011; Mazzaglia et al. 2012; Butler et al. 2013; Mc Cann et al. 2013), and very few strains which have been isolated during past epidemics. With the aim to broaden the comparison by including strains of past epidemics, in this study we have compared the Psa 1, Psa 2 and Psa 3 populations with a larger number of Psa strains isolated from *A. deliciosa* and *A. chinensis* in Japan during 1984–2011. We applied repetitive-sequence PCR typing using ERIC, BOX and REP primer sets, a technique widely used for inferring strain relationships between the pathovars of *P. syringae*.

We report the occurrence of several lineages within the pathovar, all of which are capable of effectively colonizing and infecting the host plant at a rate identical to the other Psa populations described so far. The occurrence of a large number of Psa lineages has also been confirmed by the differential detection of 23 type III effector genes. The strain assessment for tolerance to arsenic and for the presence of genes of the phaseolotoxin *argK-tox* cluster and coronatine also reveals differing behaviours among the Psa lineages. Consequently, the basis of the current naming system of Psa populations on numbering and the presence or absence of phytotoxins should be reconsidered.

## Materials and methods

### Bacterial strains

The *Pseudomonas* strains used in this study are listed in Table 1. All strains were cultured on nutrient agar

**Table 1** List of *Pseudomonas syringae* pv. *actinidiae* (Psa) strains isolated from past and current epidemics used in this study

| Strain name | Host plant- cultivar | Year of isolation | Origin | Past (P) or current (C) pandemia | Toxin detection | | Rep-PCR lineage |
|---|---|---|---|---|---|---|---|
| | | | | | PHAS | COR | |
| Psa NCPPB $3739^T$ | *Actinidia deliciosa* Hayward | 1984 | Japan | P | + | – | 3 |
| Psa SUPP 1753 | *A. deliciosa* Hayward | 1984 | Japan | P | + | – | 7 |
| Psa SUPP 1747 | *A. deliciosa* Hayward | 1986 | Japan | P | + | – | 5 |
| Psa SUPP 1758 | *A. deliciosa* Hayward | 1986 | Japan | P | + | – | 8 |
| Psa SUPP 765 | *A. deliciosa* Hayward | 1987 | Japan | P | – | – | 1 |
| Psa SUPP 777 | *A. deliciosa* Hayward | 1987 | Japan | P | – | – | 1 |
| Psa SUPP 975 | *A. deliciosa* Hayward | 1988 | Japan | P | – | – | 1 |
| Psa SUPP 1459 | *A. deliciosa* Hayward | 1991 | Japan | P | + | – | 3 |
| Psa SUPP 1765 | *A. deliciosa* Hayward | 1995 | Japan | P | + | – | 6 |
| Psa SUPP 1766 | *A. deliciosa* Hayward | 1995 | Japan | P | + | – | 1 |
| Psa SUPP 2311 | *A. deliciosa* | Nk (before 2000) | Japan | P | – | – | 9 |
| Psa SUPP 2070 | *A. deliciosa* | 2001 | Japan | P | + | – | 1 |
| Psa SUPP 2072 | *A. deliciosa* | 2001 | Japan | P | + | – | 4 |
| Psa SUPP 2211 | *A. deliciosa* | 2002 | Japan | P | + | – | 2 |
| Psa SUPP 2726 | *A. deliciosa* Hayward | 2009 | Japan | P | + | – | 3 |
| Psa SUPP 2818 | *A. chinensis* Rainbow red | 2011 | Japan | P | + | – | 5 |
| Psa SUPP 2820 | *A. deliciosa* Hayward | 2011 | Japan | P | + | – | 1 |
| Psa NCPPB 3871 | *A. deliciosa* Hayward | 1992 | Italy | P | – | – | 3 |
| Psa KN.2 | *A. deliciosa* | 1997 | South Korea | P | – | + | 10 |
| Psa CRA FRU8.43 | *A. chinensis* Hort16A | 2008 | Italy | C | – | – | 11 |
| Psa CRA FRU 8.56 | *A. deliciosa* Hayward | 2009 | Italy | C | – | – | 11 |
| Psa CRA FRU 12.57 | *A. deliciosa* Hayward | 2010 | Italy | C | – | – | 11 |
| Psa CRA FRU 12.37 | *A. chinensis* JinTao | 2010 | Italy | C | – | – | 11 |
| Psa CRA FRU 12.50 | *A. chinensis* A Hort16 | 2011 | Italy | C | – | – | 11 |
| Psa CRA FRU 13.02 | *A. deliciosa* Hayward | 2012 | Italy | C | – | – | 11 |
| Psa CRA FRU 16.45 | *A. chinensis* Hort16A | 2013 | Italy | C | – | – | 11 |
| Psa CL 1- | *A. deliciosa* Hayward | 2013 | Chile | C | – | – | 11 |
| Psa CL 5 | *A. deliciosa* Hayward | 2013 | Chile | C | – | – | 11 |
| Psa CL 6 | *A. deliciosa* Hayward | 2013 | Chile | C | – | – | 11 |
| Psa CL 7 | *A. chinensis* Kis Y374 | 2013 | Chile | C | – | – | 11 |

**Table 1** (continued)

| Strain name | Host plant- cultivar | Year of isolation | Origin | Past (P) or current (C) pandemia | Toxin detection | | Rep-PCR lineage |
|---|---|---|---|---|---|---|---|
| | | | | | PHAS | COR | |
| Psa CL 13 | *A. deliciosa* Matua | 2013 | Chile | C | – | – | 11 |
| Psa CL 23 | *A. deliciosa* Matua | 2014 | Chile | C | – | – | 11 |
| Psa CL 15 - C | *A. deliciosa* Hayward | 2013 | Chile | C | – | – | 11 |
| Psa IVIA 3729.2 | *A. deliciosa* | 2011 | Spain | C | – | – | 11 |
| Psa 36.32 | *A. deliciosa* | 2011 | France | C | – | – | 11 |
| Psa 354 | *A. deliciosa* | 2011 | Portugal | C | – | – | 11 |
| Psa ICMP 18801 | *A. chinensis* | 2011 | New Zealand | C | – | – | 11 |
| Psa ICMP 18884 | *A. deliciosa* | 2010 | New Zealand | C | – | – | 11 |
| Psa M23 | *A. chinensis* | 2010 | China | C | – | – | 11 |
| Psa M218 - | *A. chinensis* | 2010 | China | C | – | – | 11 |

It is reported their origin, host plant, year of isolation, the occurrence of phaseolotoxin (PHAS) or coronatine (COR) phytotoxins and the lineage numbering inferred by repetitive-sequence PCR analysis

Psa: *P. syringae* pv. *actinidiae*. Nk: not known. $^{T}$: pathovar type-strain

CL: Culture Collection of National Plant Protection Service, Santiago, Chile

CRA-FRU: Culture Collection of C.R.A.-Centro di ricerca per la Frutticoltura, Roma, Italy

ICMP: International Collection of Microorganisms from Plants, Lincoln, New Zealand

IVIA: Instituto Valenciano de Investigaciones Agraria, Moncada-Valencia, Spain

NCPPB: National Collection of Plant Pathogenic Bacteria, York, United Kingdom

SUPP: Culture Collection of the Shizuoka University, Shizuoka, Japan

(Oxoid) supplemented with 3 % nutrient sucrose agar (NSA) and incubated at 23–25 °C.

Repetitive-sequence PCR fingerprinting

All Psa strains obtained from different geographical areas and in different years from both *A. deliciosa* and *A. chinensis* were subjected to repetitive-sequence PCR (rep-PCR) fingerprint analysis using BOX, ERIC and REP primer sets (Louws et al. 1994; Ferrante and Scortichini 2009; 2010; 2015). Genomic DNA was extracted with the GeneElute Bacterial Genomic DNA kit (Sigma Aldrich, St Louis, MO, USA). DNA concentrations were estimated by measuring the absorbance at 260 and 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific. Wilmington, DE, USA). Amplicons were separated by electrophoresis on 2 % (*w*/*v*) agarose gels in 0,5X TAE buffer at 50 V and 4 °C for 16 h (Ferrante and Scortichini 2010; 2015). Cluster analysis was performed on a similarity matrix and was subjected to the unweighted pair group method with the arithmetic average (UPGMA) clustering algorithm and the Dice's coefficient using the web tools available at www.pubmlst.org.

Detection of type III secretion system effector genes

The putative presence of 23 type III effector genes in Psa strains was evaluated by PCR using the primers described elsewhere (Ferrante and Scortichini 2010; Ferrante and Scortichini 2011; 2015). The presence or absence of a band of the expected size indicated the presence or absence of the gene in the tested strain (Ferrante and Scortichini 2010; 2011; 2015). Cluster analysis was performed on a similarity matrix and was subjected to the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm and the Dice's coefficient using www.pubmlst.org.

Detection of phaseolotoxin and coronatine phytotoxins

The presence of the *cfl* gene coding for coronatine and of fragments of the *argK-tox* gene cluster coding for phaseolotoxin was evaluated by PCR amplification in all of the Psa cultures assessed in this study. The PCR reactions were performed as described previously (Bereswill et al. 1994; Dos Marques et al. 2000; Sawada et al. 2002). *P. syringae* pv. *tomato* DC 3000, Psa NCPPB 3739$^T$ and Psa CRA-FRU 8.43 were used as controls. The presence or absence of a band of the expected size was taken as indication of the presence or absence of the gene fragment in the genome of the tested strains.

Tolerance to arsenic

Arsenate-resistant proteins were found in one Psa strain, namely, ICMP 18744=CRA-FRU 11.41 (Butler et al. 2013). This strain was isolated in Italy during the current kiwifruit epidemic (Ferrante and Scortichini 2010). To expand the knowledge of the resistances and tolerances of Psa strains to arsenic, an in vitro assay was carried out to assess the behaviour of this *P. syringae* pathovar to sodium arsenite ($AsO_2Na$). Sodium arsenite solution (Sigma-Aldrich, St. Louis, USA) at 0.15, 0.25 and 0.35 mM was added to the following *Pseudomonas* minimal medium (Andolfi et al. 2014): 1.0 g/l ammonium dihydrogen orthophosphate, 0.2 g/l potassium chloride, 0.2 g/l magnesium sulphate, 1.0 g/l peptone, 10.0 g/l glucose, and 15 g/l technical agar. For each strain, a suspension of $1–2\times10^6$ cfu/ml was plated onto the medium containing sodium arsenite, and the colony count was performed 48 h after the distribution of the suspension on the plate. The Psa response to sodium arsenite was compared with that of other phytopathogenic pseudomonads, namely, *P. viridiflava* CRAFRU 10.34, *P. syringae* pvs *lachrymans* PD 580, *morsprunorum* RIPF 77, *syringae* CRAFRU 10.31, *theae* NCPPB 2598 and *tomato* DC 3000. The experiment was performed in duplicate. The presence of *arsH* and *arsR* genes of the *ars* operon, which is involved in arsenic resistance through an ATP-dependent efflux mechanism, was also checked. The primers were designed using the PRIMER3 program based on the sequences of Psa CRA-FRU 8.43 genome (Marcelletti et al. 2011); *arsH* forward:5′-TCCTGCTGCTTTATGGGTCA-3′; *arsH* reverse: 5′-CTTCCGACCACAACACCATC-3′; *arsR* forward: 5′-CTGTGCATTGCTGGATGGTT-3′; *arsR* reverse: 5′-GCTGTGTGGCGATGAGTAAG-3′. All PCR reactions were performed in a Bio-Rad MJ Mini thermal cycler with the following cycling conditions: denaturation at 95 °C for 5 min, 35 cycles of 30 s of annealing at 58 °C and extension at 72 °C for 1 min, and 5 min of final extension at 72 °C. Amplification products were separated in 1 % agarose gels and visualized by a Bio-Rad Gel Logic 100 UV transilluminator.

## *P. syringae* pv. *actinidiae* growth in kiwifruit leaves

To compare the growth of the different Psa populations, one-year-old, potted greenhouse-grown *A. deliciosa* cv. Hayward plants were transferred during the spring to a growth chamber set at 95 % humidity and 21±1 °C one week before inoculation. The plants were inoculated with the following strains: SUPP 777, SUPP 1753, SUPP 1758, SUPP 1765, SUPP 2072, SUPP 2211, SUPP 2311, SUPP 2818, NCPPB 3739$^T$, KN.2, and CRA-FRU 8.43. These strains represent the 11 lineages revealed by rep-PCR analysis. For inoculation, strains were grown on NSA for 48 h at 23–25 °C. A single colony was suspended at a concentration of 1–2× $10^6$ cfu/ml in sterile saline (SS) (0.85 % NaCl in distilled water). Inoculations were performed as previously described by Marcelletti et al. (2011) and Ferrante and Scortichini (2015). Briefly, leaf areas of approximately 1 cm in diameter were inoculated by pressing a needleless sterile syringe against the leaf to infiltrate the bacteria. For each strain, 10 leaves from five independent plants (i.e., two leaves per plant) were inoculated in four sites. Control plants were treated with only SS. To determine bacterial growth *in planta*, 2, 8 and 21 days after inoculation, four leaf disks of approximately 0.5 cm in diameter were sampled and ground in 1 ml of sterile saline. Serial 10-fold dilutions were spotted onto NSA medium. Colonies were counted two days after incubation at 23–25 °C to determine the putative Psa concentration in cfu/ml. At each sampling, identity confirmation was achieved by performing rep-PCR (Ferrante and Scortichini 2009; 2010; 2015) using Psa CRA-FRU 8.43 as a positive control. The standard deviation of log-transformed growth data was calculated using SPSS software package, version 20.0 (SPSS Inc.). Re-isolation and confirmation of strain identity from plants were performed using previously established procedures (Ferrante and Scortichini 2009; Ferrante and Scortichini 2010; 2015).

# Results

## Repetitive-sequence PCR fingerprinting

Rep-PCR fingerprinting performed with ERIC, BOX and REP primer sets, clearly showed a relevant diversity among the Psa strains isolated in Japan between 1984 and 2011 compared to the uniformity of the strains obtained from all other combined areas of the world (including Italy, Spain, France, Portugal, New Zealand, Chile, and China) and isolated during the current pandemic (i.e., 2008 onward). Representative gels showing rep-PCR products, including all strains isolated in Japan, are presented in Fig. 1a-c, and the corresponding concatenated UPGMA dendrogram is shown in Fig. 2. Differences among banding patterns between strains are clearly visible with each primer set. The pathovar-type strain, NCPPB 3739$^T$, which was isolated in 1984 in Japan, shows a remarkable similarity with only three

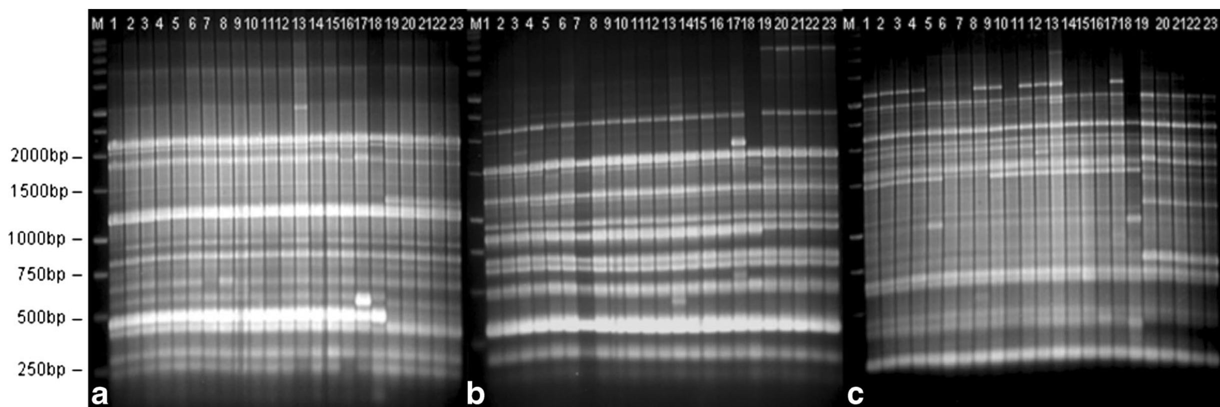


**Fig. 1** Representative repetitive-sequence PCR fingerprinting patterns for genomic DNA of *Pseudomonas syringae* pv. *actinidiae* strains isolated from past and current epidemics of bacterial canker of kiwifruit, and obtained using BOX (**a**), ERIC (**b**) and REP (**c**) primer sets (see also Table 1). M. Molecular size marker 1-kb DNA ladder (Promega). Lane *1*: SUPP 777; lane *2*: SUPP 1766; lane *3*: SUPP 2211; lane *4*: SUPP 2820; lane *5*: SUPP 1753; lane *6*: SUPP 2818; lane *7*: SUPP 1747; lane *8*: SUPP 1765; lane *9*: SUPP 765; lane *10*: SUPP 1459; lane *11*: SUPP 975; lane *12*: SUPP 2070; lane *13*: SUPP 2072; lane *14*: SUPP 2726; lane *15*: NCPPB 3739$^{T;}$ lane *16*: SUPP 1758; lane *17*: SUPP 2311; lane *18*: KN. 2; lane *19*: CRA-FRU 8.43; lane *20*: IVIA 3729.2; lane *21*: ICMP 18884; lane *22*: M 218; lane *23*: CL 1

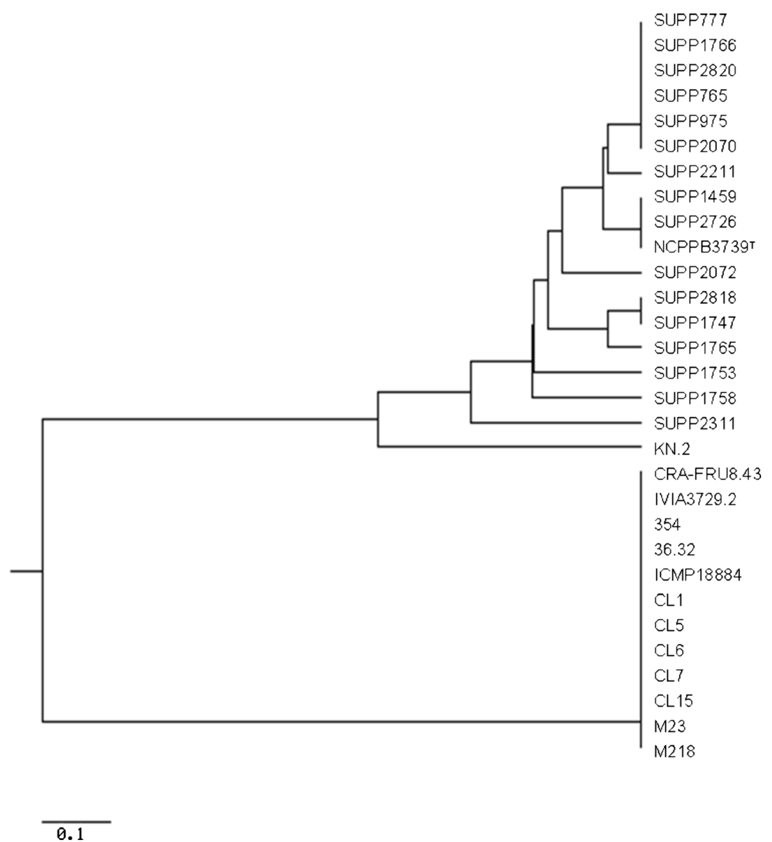


**Fig. 2** Dendrogram of relationships among 30 representative *Pseudomonas syringae* pv. *actinidiae* strains isolated from past and present epidemics of kiwifruit bacterial canker, including all strains isolated in Japan, inferred by UPGMA analysis and the Dice's coefficient of the concatenated repetitive-sequence PCR fingerprinting data set performed with BOX, ERIC and REP primer sets. The scale at the bottom indicates the degree of genetic similarity among the strains. For all Psa strains, the lineage numbering is shown in Table 1

other Psa strains, namely, SUPP 1459 and SUPP 2726, which were isolated in Japan from *A. deliciosa* cv. Hayward in 1991 and 2009, respectively and with NCPPB 3871 which was isolated in Italy in 1992. Three of the four strains lacking the *argK-tox* cluster (see below) were grouped with other strains that possess it. A total of nine different patterns were noted among the Psa strains isolated in Japan over the 27 years, none of which belong to the current pandemic lineage (Fig. 2). The rep-PCR fingerprinting once more confirms the uniqueness of the KN.2 strain isolated in South Korea, which shows a distinct banding pattern similar to those shown by the Japanese strains (Figs. 1 and 2). No qualitative differences in banding patterns were revealed among the Psa strains isolated from different continents during the current pandemic of kiwifruit bacterial canker (Figs. 1 and 2).

## Detection of type III secretion system effector genes

The PCR amplification of 23 type III effector genes revealed differences among the Psa strains (Fig. 3). All strains of the current pandemic of kiwifruit bacterial canker share the same repertoire of effector genes, whereas the Psa strains isolated from Japan during the past epidemics showed differences. In particular, the *hopA1* effector gene was never detected in the strains isolated during past epidemics in Japan, Italy or South Korea, whereas *hopAS1* and *hopP1* were not detected in SUPP 1758 and in SUPP 2820, respectively. Three strains, namely, SUPP 2726 and SUPP 2818 isolated from Japan, and KN.2 isolated from South Korea, showed a distinct repertoire of type III effector genes (Fig. 3).

## Detection of phaseolotoxin and coronatine phytotoxins

Of all of the 40 Psa strains tested here, only KN.2 from South Korea showed the presence of *cfl* gene coding for coronatine production. Concerning the presence of genes of the *argK-tox* cluster coding for phaseolotoxin, we found differences among the strains isolated in Japan during 1984–2011. In fact, the majority of the strains showed the presence of PCR products related to

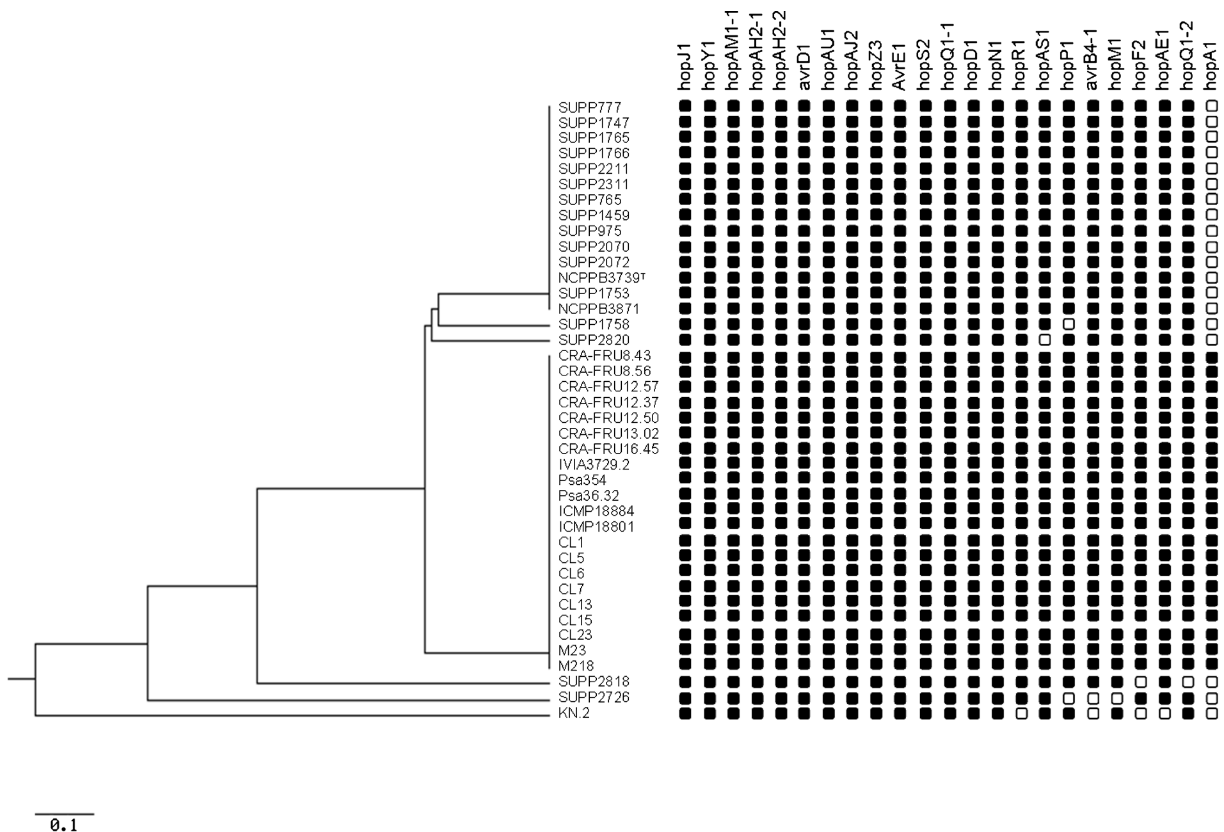


**Fig. 3** PCR detection of presence/absence of type III secretion system effector protein genes in 40 *Pseudomonas syringae* pv. *actinidiae* strains isolated from past and current epidemics of kiwifruit bacterial canker worldwide, and the corresponding dendrogram of strain relationships inferred with UPGMA and the Dice's coefficient. *Filled squares* represent presence and *open square* represent absence. The *scale at the bottom* indicates the degree of genetic similarity among the strains

fragments of the phaseolotoxin cluster (Table 1 and Fig. 4); however, four of them, namely, SUPP 765 (isolated in 1987), SUPP 777 (isolated in 1987), SUPP 975 (isolated in 1988), and SUPP 2311 (year of isolation unknown), did not show any signal of amplification for such cluster fragments (Fig. 4). None of the strains from the current pandemic showed PCR products indicating the presence of coronatine or phaseolotoxin (Table 1).

## Tolerance to arsenic

The susceptibility of Psa and other phytopathogenic pseudomonads to sodium arsenite is shown in Fig. 5. The assay conducted showed large variability among the Psa strains concerning the tolerance to sodium arsenite. In fact, some strains, including all of the strains isolated in Chile, the two strains from New Zealand and the M 23 strain isolated in China, showed growth inhibition upon the addition of any dose supplemented to the medium. In contrast, most Psa strains isolated in Italy during the current epidemic of kiwifruit bacterial canker were tolerant to 0.15–0.35 mM $AsO_2Na$. Other Psa strains of the current epidemic obtained from France, Portugal, and Spain and one strain isolated in Italy from *A. chinensis* cv. Jin Tao, namely, CRA-FRU 12.37, showed high sensitivity to 0.35 mM doses of the compound. All of the strains isolated in Japan, South Korea and Italy (1992) were inhibited at doses higher than 0.15 mM sodium arsenite. Remarkably, one Psa strain isolated in China, M 218, which was isolated from *A. chinensis*, was tolerant to all of the doses tested. Similarly, the *P. viridiflava* CRAFRU 10.34, *P. syringae* pvs *lachrymans* PD 580, *syringae* CRAF RU 10.31 and *theae* NCPPB 2598 strains herein

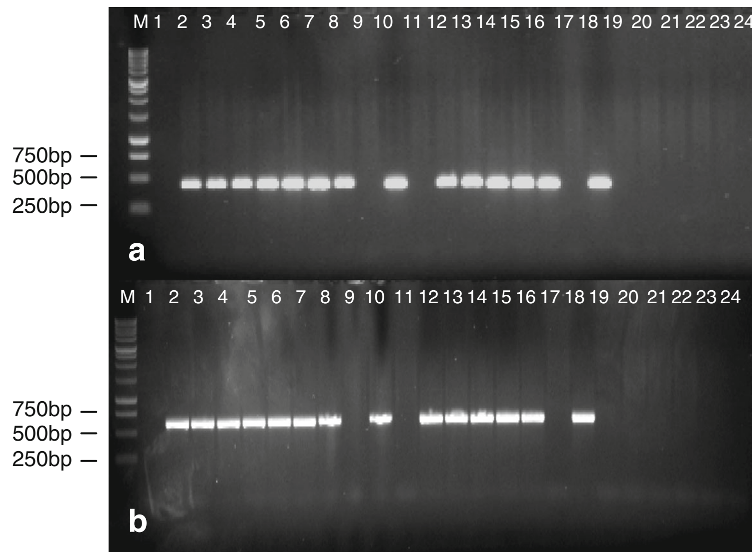


**Fig. 4** Representative gels for the detection of gene fragments (**a**: Dos Marques et al. (2000); **b**: Sawada et al. (2002) of phaseolotoxin in *Pseudomonas syringae* pv. *actinidiae* strains isolated from past and present epidemics of kiwifruit bacterial canker to *Actinidia deliciosa* and *A. chinensis*. M: molecular size marker 1-kb ladder (Promega). Lane *1*: SUPP 777; lane *2*: SUPP 1766; lane *3*: SUPP 2211; lane *4*: SUPP 2820; lane *5*: SUPP 1753; lane *6*: SUPP 2818; lane *7*: SUPP 1747; lane *8*: SUPP 1765; lane *9*: SUPP 765; lane *10*: SUPP 1459; lane *11*: SUPP 975; Lane *12*: SUPP 2070; lane *13*: SUPP 2072; lane *14*: SUPP 2726; lane *15*: NCPPB 3739$^T$; lane *16*: SUPP 1758; lane *17*: SUPP 2311; lane *18*: NCPPB 3871; lane *19*: CRAFRU 8.43; lane *20*: CL 1; lane *21*: CL 5; lane *22*: CL 6; lane *23*: CL 13; lane *24*: CL 15

assessed showed tolerance, whereas *P. s.* pvs *morsprunorum* RIPF 77 and *tomato* DC 3000 showed higher sensitivity to sodium arsenite (Fig. 5). For all Psa strains, the presence of the *arsR* gene of the *ars* operon coding for resistance to arsenic was detected using a PCR assay. *ArsH* yielded an amplicon of 702 bp, whereas *arsR* an amplicon of 684 bp. The *arsH* gene was only found in the strains of the current epidemics of kiwifruit bacterial canker isolated in Europe, in one strain from China, and in *P. viridiflava* CRA-FRU

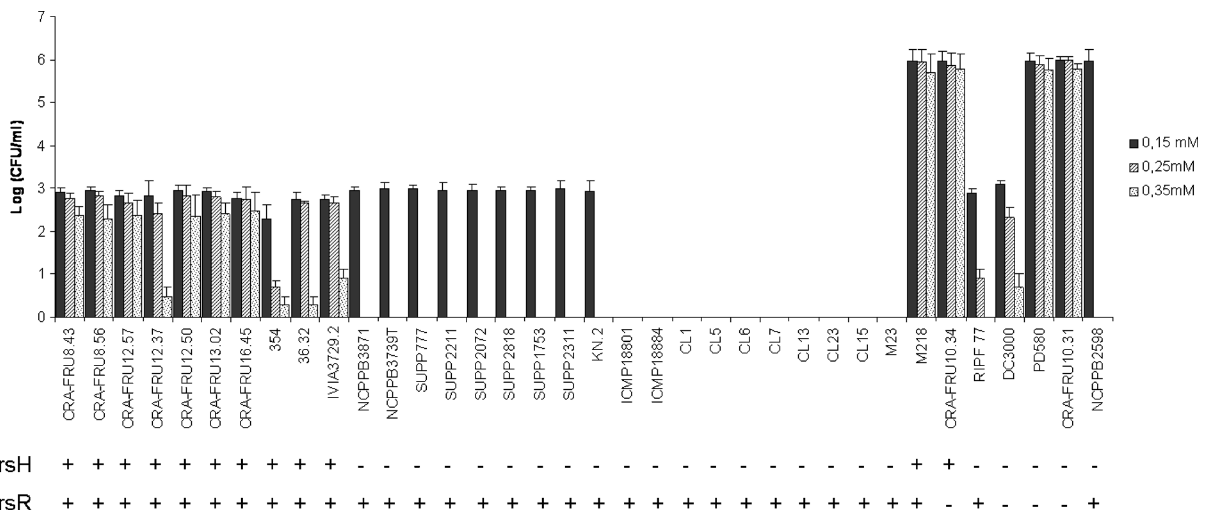


**Fig. 5** Tolerance to sodium arsenite ($AsO_2Na$) shown by *Pseudomonas syringae* pv. *actinidiae* strains of past and present epidemics of kiwifruit bacterial canker and some other phytopathogenic pseudomonads (see also Table 1 and Materials and Methods). The concentrations used in the assay are indicated at the right of the figure. The detection of *arsH* and *arsR* genes of the *ars* operon coding for resistance to arsenic is also reported. Bars represent standard deviation calculated on two replications

10.34. It was absent in all strains of past epidemics and in Psa strains of the current epidemic that were isolated in Chile and New Zealand and in another strain from China and the other phytopathogenic pseudomonads tested herein (Fig. 5).

### Growth in kiwifruit leaves

The growth in kiwifruit cv. Hayward leaves of representative Psa strains of the 11 different lineages inferred using rep-PCR typing, including the pandemic CRA-FRU 8.43, was assessed. All strains actively multiplied during the 21 days of the experiment without showing substantial differences in growth trends. All strains incited symptoms of bacterial canker (i.e., leaf spotting) from where they were re-isolated.

## Discussion

This study demonstrates a genetic variability within Psa strains that is remarkably higher than those previously reported (Ferrante and Scortichini 2010; 2015; Mazzaglia et al. 2012; Butler et al. 2013; Mc Cann et al. 2013). It is likely that the reason for the greater observed variability is that former studies investigated Psa strains isolated primarily from the current pandemic kiwifruit bacterial canker and included very few strains from the past epidemics. Therefore, the Psa strains from current global outbreaks resulted in highly similar genetic profiles despite the fact that they have been isolated from different continents. By using rep-PCR with BOX, ERIC and REP primer sets, we found a relevant genetic diversity within the Psa strains isolated from *A. deliciosa* in Japan during 1984–2011. In contrast, Butler et al. (2013) and Mc Cann et al. (2013) analysed both the housekeeping genes and the genome of Japanese strains isolated during 1984–1988 but did not observe such a striking genetic variability as that seen for the Psa strains isolated afterward. The occurrence of genetic differentiation within Psa strains isolated in Japan was confirmed by the possible absence or mutation of the phaseolotoxin gene cluster, *argK-tox*, revealed in four of the 17 strains tested and isolated in 1987–1988, namely SUPP 765, SUPP 777, SUPP 975 and SUPP 2311. To date, the presence of such phytotoxin has been retained as a basic feature for differentiating the Psa 1 population from the other three Psa populations that lack this factor (Psa 2, Psa 3 and Psa 5).

Therefore, it appears that in Japan, during a period of 27 years, different lineages of the pathogen evolved to infect *A. deliciosa* cv. Hayward. The gain and/or loss of gene(s) in the bacterial genome (Ochman and Moran 2001) could partly explain such a differentiation. In fact, it has been shown that the acquisition of the *argK-tox* cluster for phaseolotoxin production was acquired in the Psa strains through lateral gene transfer (Sawada et al. 1999) and that the acquisition was quite recent and probably derived from bacterial species distantly related to *P. syringae* (Sawada et al. 2002). Our study shows that, similar to *P. syringae* pv. *phaseolicola* (Oguiza et al. 2004), Psa strains that are isolated in the same country from the same host plant can coexist with multiple genetic lineages distinguishable by the presence or absence of the *argK-tox* cluster or, alternatively, by its gene mutation. According to rep-PCR typing, the four strains were grouped into different clusters. Psa strains lacking this cluster have also been recently isolated in Japan from *A. chinensis* cv. Hort 16A since 2010 (Sawada et al. 2014). Remarkably, it has been shown that the lack of phaseolotoxin, a bacterial virulence factor, does not affect the overall aggressiveness of these Psa lineages to both *A. chinensis* and *A. deliciosa* (Marcelletti et al. 2011). Similar results were reported with a phaseolotoxin-negative mutant of Psa (Tamura et al. 2002).

Additionally, the detection of 23 type III effector genes revealed differences between Psa strains of past and current epidemics of kiwifruit bacterial canker. In particular, all strains of the current pandemic showed the same pattern of effectors, whereas higher variability was found in the effector repertoires of strains of past epidemics isolated in Japan, Italy and South Korea. Interestingly, *hopA1* was never detected in the Psa strains of the past epidemics, but it was present in all strains of the current pandemic, which was also reported by McCann et al. (2013). This effector has protein structural motifs conserved both in animal and plant pathogens (Janjusevic et al. 2013) and is capable of suppressing the effector-triggered immunity of the host plant (Guo et al. 2009). The question of whether this single effector plays a fundamental role in the relative aggression shown by the Psa strains of the current pandemic remains to be determined.

Butler et al. (2013) found that one Italian and one Chinese Psa strain isolated during the current pandemic was tolerant to 5 mM sodium arsenate; by contrast, a 0.5 mM dose of the compound was lethal to another Psa

strain recently isolated in New Zealand. By broadening the scope of the assay, we expose the variability of Psa strain resistance to arsenic sodium salts. In fact, sodium arsenite is more toxic than sodium arsenate, so by using sodium arsenite we found that some strains of the current pandemic that were isolated in Chile and New Zealand and one strain from China were not tolerant at all; strains from the current global outbreak that were isolated in Europe were moderately tolerant, whereas the strains from past epidemics tolerated only the lowest amount tested, 0.15 mM. Remarkably, one strain isolated in China was tolerant to 0.35 mM. Tolerance to arsenic was also observed in some strains of phytopathogenic pseudomonads, such as *P. viridiflava*, *P. s.* pv. *lachrymans*, *P. s.* pv. *syringae* and *P. s.* pv. *theae*. It should also be noted that the tolerance threshold found here is lower than the tolerance values of the sodium arsenite-resistant bacteria that were isolated from the root system of plants grown in an arsenic-polluted soil, which ranged from 3 to 20 mM (Shagol et al. 2014). However, no other data about this feature of plant pathogenic bacteria are available, whereas arsenic-resistant strains have been found and characterized in environmental pseudomonads such as *P. aeruginosa* (Cai et al. 1998), *P. fluorescens* (Prithivirajsingh et al. 2001) and *P. putida* (Paez-Espino et al. 2015). To note that all Psa strains possess *arsR*, the first gene within the *ars* operon involved in arsenic resistance that is transcribed after the cell came into contact with arsenic (Wang et al. 2006). However, the selective advantage, if any, conferred by resistance/tolerance to arsenic in phytopathogenic pseudomonads remains to be elucidated.

Collectively, these data raise questions regarding the suitability of the naming of Psa populations, which is currently based on numbering according to the presence/absence of phaseolotoxin and/or coronatine. In fact, this study shows that some Psa strains from past epidemics of kiwifruit bacterial canker isolated in Japan from *A. deliciosa*, in contrast to the pathovar-type strain NCPPB 3739$^T$ isolated in the same country from the same host and period, does not have such a gene. Recently, another Psa population, namely Psa 5, which is genetically different from the Psa 1, Psa 2 and Psa 3 populations and do not have the phaseolotoxin cluster, has been isolated in Japan from *A. chinensis* (Sawada et al. 2014). It seems that, similar to other *P. syringae* pathovars, such as *lachrymans*, *phaseolicola*, *pisi* and *syringae* (Scortichini et al. 2003; Cirvilleri et al. 2007; Olczak-Woltman et al. 2007; Kaluzna et al. 2010; San José et al. 2010; Martin-Sanz et al. 2012), the pathovar *actinidiae* is also composed of several genetically different lineages capable of causing infection in *Actinidia* spp. in various areas of the world, and the precise distinctiveness of these strains based on a few indeterminate features, such as the presence/absence of phaseolotoxin gene cluster, is quite arbitrary.

Additionally, the current pandemic Psa 3 population is not equable. In fact, based on the genomic analyses of horizontally transmissible genomic islands, Butler et al. (2013) noted that three slightly different Psa clones reached Italy, New Zealand and Chile, most likely deriving from China, and caused widespread damages. However, from a phytosanitary legislation point of view, these clones can be precisely detected as a unique population (Gallelli et al. 2014). Because all major areas of kiwifruit cultivation of the world are currently affected by such a population, we can continue to refer to this as Psa 3 or pandemic Psa and refer to the others by quoting the geographic origin, host and year of isolation and some basic (molecular and/or phenotypic) characteristics rather than resorting to a numbering system. Finally, because all Psa populations are virulent to *A chinensis* and *A. deliciosa*, the definition of Psa 3 as Psa-V (i.e., virulent) is unnecessary.

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